Journal of Chromatography, 363 (1986) 337–344
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 721

SEPARATION OF LISSAMINE RHODAMINE B SULFONYL DERIVATIVE OF AMINO ACIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND THIN-LAYER CHROMATOGRAPHY

ROBERT M. METRIONE
Department of Biochemistry, Jefferson Medical College, Thomas Jefferson University, Philadelphia, PA 19107 (U.S.A.)
(Received April 11th, 1986)

SUMMARY

The effectiveness of laryl chloride (Lissamine rhodamine B sulfonyl chloride) as a reagent for labeling free amino groups of amino acids, peptides and proteins has been demonstrated. Laryl amino acids are bright red compounds which absorb light with a maximum at a wavelength of 560 nm and emit light with a maximum at a wavelength of 595 nm. On thin-layer plates, fluorescence is about 125 times more sensitive than visual observation of the colored spots and it was possible to observe laryl amino acids in amounts as low as 400 fmol, about half the amount which is needed for dansyl amino acids. The colorimetric thin-layer methods are as sensitive as the dabsyl chloride method and do not require development with hydrochloric acid fumes. A high-performance liquid chromatographic method was developed for the separation of the laryl amino acids and the usefulness of the technique was demonstrated on small peptides.

INTRODUCTION

Lissamine rhodamine B sulfonyl (laryl) chloride has been used to prepare fluorescent protein conjugates for some time[1]. Since the functional group is a sulfonyl chloride, it is likely that the reaction with proteins is analogous to that of dabsyl chloride[2] and dansyl chloride[3], in that the reagent would react with primary and secondary amino groups to form stable products. Because the reagent absorbs light in the visible range, it has potential as an amino-terminal reagent which could be analyzed by thin-layer or high-performance chromatography. Furthermore, because it is intensely fluorescent, it has the possibility of being measurable at very low concentrations.

With the recent emphasis on the determination of the characteristics of very small amounts of protein, techniques of high sensitivity have become increasingly important. One of the most widely used reagents for the determination of the amino-terminal residues of proteins is dansyl chloride (5-dimethylamino-naphthalene-1-sulfonyl chloride). This compound, when analyzed by thin-layer chromatography[3],

0021-9673/86/$03.50

has been reported to allow detection to at least 10 pmol of amino acid[4]. This high sensitivity is due to the intense fluorescence of dansyl amino acids. However, for thin-layer chromatography, the use of colored derivatives has an advantage over fluorometric procedures in that special lighting is not required to see the spots on the thin-layer plates, and because movement of the spots can be observed as it occurs, allowing chromatography to be stopped as soon as separations are sufficient to yield the desired information. In addition, when performing high-performance liquid chromatography (HPLC), solvent restrictions are less stringent than when using UV absorbing materials since UV-absorbing contaminants in the solvents do not interfere with peak identifcation.

In 1975 Lin and Chang[2] introduced dabsyl chloride (4-dimethylaminoazobenzene-4′-sulfonyl chloride), a strongly colored compound, as an amino-terminal reagent. When combined with thin-layer chromatography on polyamide sheets, it has been possible to observe 10–100 pmol of dabsyl amino acid[5]. However, for full sensitivity to be realized, the completed thin-layer chromatogram must be exposed to hydrochloric acid fumes which causes conversion of the orange spots to a more intense red color. Thus, if working at the extremes of sensitivity, it is not possible to observe the movement of the spots.

This paper demonstrates the reaction of Lissamine rhodamine B sulfonyl chloride with amino groups and demonstrates the usefulness of this compound as a reagent for amino terminal analysis and amino acid analysis of peptides which combines the advantages of both dabsyl chloride and dansyl chloride, and has certain advantages over each of these reagents.

EXPERIMENTAL

Materials

Laryl chloride was purchased from Eastman-Kodak and was used without further purification. Dansyl chloride was from Pierce, and all dansyl amino acids were from Mann Reserch Labs. The dabsyl chloride and dabsyl amino acids were prepared by the methods of Lin and Chang[2]. All solvents were reagent grade except those which were used for HPLC which were HPLC grade (Fisher). Methyl orange was obtained from Fisher Scientific. The silica gel thin-layer plates were Eastman 13179 and the polyamide sheets were obtained from Brinkman.

Methods

The laryl amino acids were prepared from laryl chloride by a method very similar to that employed for the preparation of dabsyl amino acids[2]. For thin-layer chromatography, amino acid (1 mg/100 μl) in 0.1 *M* sodium hydrogen carbonate at pH 9.0 was mixed with 100 μl of laryl chloride (100 μg) in a 6 × 50 mm tube. For HPLC the amino acids were prepared at a concentration of 0.2 nmol per 10 ml in 40 m*M* lithium carbonate pH 9.5 and 100 μl of this solution was mixed with 50 μl of laryl chloride (5 mg/ml in acetone). The pH of all reaction mixtures was within 0.1 pH unit of 9.0. The tubes were sealed with parafilm and heated at either 70°C for 10 min or 50°C for 30 min. Preparations of laryl amino acids were treated with 6 *M* hydrochloric acid at 105°C for 16 h to test for stability of the derivatives under conditions employed for peptide bond hydrolysis. No changes in R_F values or inten-

sity of spots were observed upon thin-layer chromatography suggesting that the laryl amino acids were reasonably stable under these conditions.

The absorption spectra were obtained with a Beckman DBG spectrophotometer with a Houston Instruments Omnigraphic recorder. The excitation and emission spectra were taken in a Perkin-Elmer Model 650-105 fluorescence spectrophotometer with a Perkin-Elmer 56 recorder attached. Thin-layer chromatography was done on either Brinkman Polygram polyamide-6 plates or Eastman 13179 silica gel plates.

The liquid chromatography was performed on a Beckman-Altex HPLC apparatus composed of two Model 110A pumps, a Model 165 detector and a Model C-RIB integrator, or a Perkin-Elmer Model 3D chromatograph with a Model 650 fluorescence detector and a LC-75 spectrophotometric detector. The conditions were a modification of those developed by Wiedmeier *et al.*[6] for the separation of dansylated amino acids. The solvents were (A) 0.01 *M* sodium acetate buffer (pH 4.18) containing 4% dimethyl formamide and (B) acetonitrile containing 4% dimethyl formamide. A 250 × 4.6 mm column of Ultrasphere ODS was equilibrated with 25% B in A before injection. The flow-rate was 1 ml/min. After 30 s of injection the concentration of B was increased to 30% over 10 min and held at 30% for 5 min. At the end of this isocratic period, a gradient to 45% B was formed over a 45-min period. The concentration of B was then raised in a step to 90% and then isocratic for 5 min and then re-equilibration at 25% B was performed for 10 min.

RESULTS

Twenty commonly occurring amino acids were subjected to treatment with laryl chloride under conditions which had been reported for the preparation of dabsyl chloride[5]. The samples were spotted on thin-layer sheets of polyamide and developed in a number of solvent systems which had proven useful for the separation of dansyl amino acids[7]. Some of these solvent systems were capable of indicating the presence of unique amino acid derivatives, as evidenced by the presence of colored/fluorescent spots with distinctive mobilities from each amino acid reaction mixture.

After testing a number of new solvent systems on both polyamide sheets and silica gel plates, three systems were found to be particularly useful. The amino acids are listed in Table I in order of increasing R_F value in thin-layer solvent system A. By comparing adjacent amino acids in this column with R_F values in thin-layer solvents B and C, it can be seen that all of the commonly occurring amino acids can be identified by the use of these three solvent systems. For example, although laryl-Asp and laryl-Glu are not separated with solvents A and B, a clear separation occurs in system C. Similarly although laryl-Cys and laryl-Ala are not separated with solvents A and C, a good separation can be obtained in system B.

The use of laryl chloride as a qualitative amino-terminal reagent was tested by thin-layer chromatography on a number of commercially available peptides including Ala-Phe, Ala-Gly-Phe, Glu-Gly-Phe, Gly-Phe, Gly-Tyr, Gly-Lys, Val-Ala-Ala-Phe, and porcine glucagon. All of these peptides yielded fluorescent spots with R_F values corresponding to those of the laryl derivatives of the known amino-terminal amino acid.

Although 70°C for 10 min can be used as the coupling conditions, a lower

TABLE I

R_F VALUES FOR LARYL AMINO ACIDS IN VARIOUS SOLVENT SYSTEMS IN THIN-LAYER CHROMATOGRAPHY

Solvent systems: A = methanol–*n*-butylacetate–ammonium hydroxide (29%) (10:20:2); B = ethylacetate–ethanol–ammonium hydroxide (20:5:1); C = *n*-propanol–chloroform–acetic acid (99.7%) (80:20:5). A was used on silica gel plates, B and C were used on polyamide sheets.

Laryl amino acid	*R_F in solvent system*		
	A	*B*	*C*
Asp	0.08	0	0.13
Glu	0.08	0	0.26
Lys	0.11	0.09	0.76
Arg	0.15	0.04	0
Asn	0.16	0.20	0.33
Gln	0.16	0.12	0.18
His	0.17	0.15	0.65
Gly	0.18	0.21	0.33
Pro	0.18	0.22	0.53
Ser	0.18	0.21	0.24
Thr	0.18	0.33	0.50
Laryl-OH	0.19	0	0
Cys	0.23	0.07	0.04
Ala	0.25	0.39	0.04
Met	0.25	0.62	0.53
Tyr	0.25	0.20	0.27
Trp	0.27	0.30	0.29
Lev	0.29	0.72	0.74
Phe	0.31	0.06	0.58
Ile	0.32	0.76	0.79
Val	0.33	0.71	0.68
Laryl-NH_2	0.41	0.55	0.67

temperature for longer times (45°C for 2 h or 50°C for 30 min) yielded slightly more of the laryl amino acid. However, because of the shorter times of reaction, the higher temperature is much more convenient for qualitative experiments.

Although the intense color of the laryl compounds makes visual observation of thin-layer spots convenient during runs as well as while measuring R_F values after each run, much greater sensitivity can be achieved by taking advantage of the fluorescent properties of these compounds in UV light. Under these conditions the spots appear as intense orange on a purple background giving excellent color contrast which combined with the high spot intensity permits visualization at very low levels (< 1 pmol).

Since the laryl amino acids share visible color properties with dabsyl amino acids, as well as sharing the ability to fluoresce with dansyl amino acids[8], it was decided to study the relative sensitivities of these three reagents (dabsyl chloride, dansyl chloride and laryl chloride) in a thin-layer analytical system which was useful for each. Alanine was treated with the three reagents and the resulting derivative mixtures were spotted on silica gel and polyamide gel and polyamide layers in varying

amounts, and the plates were each developed in a solvent which was suitable for the particular derivative. The best systems (type of thin-layer and solvent system) for each derivative were compared for relative sensitivity.

During the development it became apparent that laryl alanine was visible at a lower concentration than dabsyl alanine. Exposure of dabsyl alanine to hydrochloric acid fumes increased the sensitivity to a level equal to that obtained with laryl alanine, *i.e.* the two methods were equally sensitive, both methods being useful down to 50 pmol of alanine derivative.

When the dansyl and laryl methods were compared by fluorescence under UV light, a much greater sensitivity was achieved than with methods based on visible color. The silica gel plates were slightly more sensitive in detecting laryl alanine than the polyamide sheets because the spots were more compact. On silica gel plates [in *n*-butanol–acetic acid (99.7%)–water (12:3:5)] laryl alanine could be seen down to 0.4 pmol, an 125-fold increase in sensitivity over the colorimetric methods. The dansyl alanine could be observed down to 0.8 pmol, the procedure being about 60 times as sensitive as the two colorimetric methods, although the practical limit of each method is somewhat higher than the value given above.

Excitation and emission spectra were determined on a preparation of laryl glycine which had been passed through a DEAE cellulose column to remove the larylic acid resulting from the hydrolysis of the sulfonyl chloride. The spectra are shown in Fig. 1. There is an excitation maximum at 560 nm and an emission maximum at 595 nm. The absorption spectrum was also determined and the absorption peak coincided with the excitation peak (560 nm).

While thin-layer chromatography can be a useful method, mainly in its low cost, simplicity and convenience, a satisfactory HPLC method offers advantages of speed and potential for quantitation. Quantitative HPLC methods for dansyl amino acids have been reported by a number of laboratories[6,9–13]. An HPLC system was therefore developed for the separation and identification of laryl amino acids (Fig. 2). This method reliably separates all of the common laryl amino acids except laryl aspartic acid. This amino acid sometimes appears as a broad flattened peak with much less height and therefore less sensitivity than other amino acids. We have no explanation for this anomalous behavior.

An additional problem with laryl aspartic acid is that it does not consistently separate from an unknown contaminant. Better results can be obtained by employing

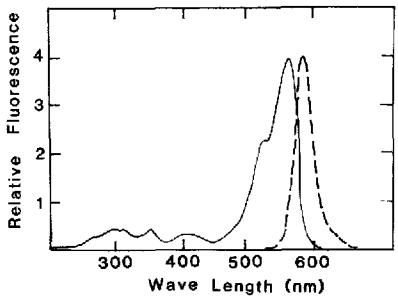

Fig. 1. The fluorescence spectra of laryl glycine. The solid line is the excitation spectrum with emission at 595 nm. The dashed line is the emission spectrum with excitation at 560 nm.

TABLE II

AMINO ACID ANALYSIS OF PEPTIDES

The value of 0 for amino acid analysis indicates that no amino acid or a trace amount was found. For N-terminal analysis by thin-layer chromatography, + indicates the presence and − the absence of a spot under the conditions of Table I. The value in parentheses is the experimentally determined number based on a value of Ala set at 1.00 for all the peptides except the tetrapeptide, in which case it was set at 2.00.

Amino acid	*Val-Ala-Ala-Phe*		*Phe-Asp-Ala-Ser-Val*		*Gly-Ala-Phe*		*Ala-Phe-Gly*	
	Amino acid analysis	*N-terminal analysis*	*Amino acid analysis*	*N-terminal analysis*	*Amino acid analysis*	*N-terminal analysis*	*Amino acid analysis*	*N-terminal analysis*
Ala	2 (2.00)	−	1 (1.00)	−	1 (1.00)	−	1 (1.00)	+
Asp	0	−	1 (0.58)	−	0	−	0	−
Gly	0	−	0	−	1 (1.00)	+	1 (1.28)	−
Phe	1 (1.16)	−	1 (1.12)	+	1 (0.75)	−	1 (0.60)	−
Ser	0	−	1 (0.86)	−	0	−	0	−
Val	1 (1.12)	+	1 (1.04)	−	0	−	0	−

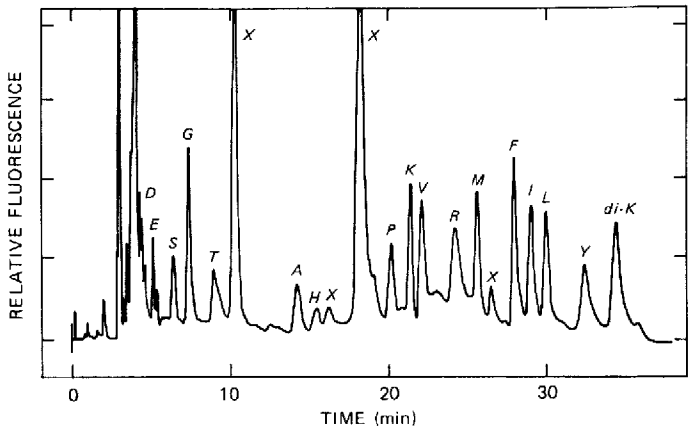

Fig. 2. HPLC of a larylated standard amino acid mixture. An amount of 2 nmol of each laryl amino acid was injected and chromatography was performed as described in the *Methods* section. Single letter designation of amino acids is used to identify peaks, X signifies major unidentified byproducts of the reaction.

a more gentle initial gradient. For this purpose, chromatography is started at 25% B for 5 min after injection. This was followed by an 8-min gradient to 30% B, followed by a 5-min isocratic hold at 30% B. A gradient to 32% B over 6 min was then performed before initiation of the gradient to 45% B. However, even under these conditions, quantitation of laryl aspartic acid is not as good as with other laryl amino acids.

The procedure is quantitative, with the peak area being proportional to concentration over the range 0.5–2.5 nmol with the absorption monitor used in these studies. Several peptides (2.5 nmol) were hydrolyzed, larylated and subjected to HPLC. The results are shown in Table II. The results show reasonable agreement with the expected structures.

The use of the method for amino-terminal analysis was demonstrated on four peptides, all of which yielded the expected peaks upon HPLC (Table II). No attempt to quantitate the amino-terminal analysis was made because it has been established that the conditions employed for hydrolysis result in the loss of dansyl groups from dansyl peptides and amino acids[9] and it is assumed that similar losses would result with laryl derivatives.

DISCUSSION

The use of colored and fluorescent sulfonyl chlorides as amino terminal reagents has been expanded over the last few years, with the advent of HPLC, to their use in amino acid analysis[6,9,14–17], making these reagents extremely useful in peptide structure studies. The most widely used of these reagents have been dansyl[3] chloride and dabsyl chloride[2]. The available methods combine speed, sensitivity and quantitation (HPLC) as well as convenience and low cost (thin-layer chromatography). Derivatization for HPLC can be either precolumn or postcolumn. The postcolumn methods require more complex equipment, *e.g.*, an additional pump, reaction coil, etc., and the required expenditure of additional funds may not be warranted in laboratories which perform a small number of analyses in a given time period. Both

dansyl and dabsyl methods are very sensitive, being useable down to the subnanomole range with the practical lower limit of usefulness probably in the range 100–300 pmol[9] because contamination of solvents and vessels becomes a problem below this range. The main disadvantage of dansyl chloride, and probably other sulfonyl chlorides such as laryl chloride for amino acid analysis, is that the reaction time is somewhat slower than that with *o*-phthalaldehyde. However, it has recently been shown that under the proper conditions dansylation is complete in 35 min at room temperature and in less time at elevated temperature[10].

One major advantage of dansylation and other similar methods is their versatility in protein chemistry. These methods permit amino terminal analysis as well as amino acid analysis and can be employed in peptide sequencing procedures such as the dansyl-Edman procedure[3]. The laryl chloride techniques described in this paper are an extension of existing sulfonyl chloride methods.

The use of laryl chloride as a reagent for analyzing the amino termini of peptides and proteins combines the advantages of both the dansyl[8] and dabsyl[4] methods and has several advantages over these techniques. The method is more versatile than either of the above techniques in that the same procedure can be used for materials which are available in relatively large amounts, with the convenience of visual observation of the color, and in those materials which are available in only small amounts, by use of fluorescence. In addition, full color development does not require any treatment of thin-layer plates as does the dabsyl method. The fluorimetric laryl method is more sensitive than the dansyl procedure.

Both thin-layer and HPLC methods are reported here. While the HPLC methods are clearly preferable for speed and potential quantitation, the thin-layer methods still are useful. Theyare cheap and convenient to perform and are useful for qualitative confirmation of results determined by HPLC[11,12].

ACKNOWLEDGEMENT

The author gratefully acknowledges Dr. Joel M. Kauffman of the Philadelphia College of Pharmacy and Science for many helpful discussions and useful suggestions.

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